IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re Patent Application of

MAERTENS et al. — Atty. Ref.: 2551-117

Serial No. 09/873,224 — TC/A.U.: 1631

Filed: June 5, 2001 — Examiner: Martinell

For: NEW SEQUENCES OF HEPATITIS C VIRUS GENOTYPES AND THEIR USE AS THERAPEUTIC

* *

February 28, 2005

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

APPEAL BRIEF

Sir:

Applicant hereby appeals the file rejection of claims 27-57, in the Office Action dated April 1, 2004, as supplemented by the comments in the Advisory Action dated October 13, 2004, and submits the present Appeal Brief pursuant to 37 CFR § 41.37.

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(1) REAL PARTY IN INTEREST

The real party in interest is N.V. Innogenetics S. A., Industriepark Zwijnaarde 7 Box 4, B-9052 Ghent, Belgium, by way of an Assignment from the applicants, recorded in the U.S. Patent and Trademark Office on January 11, 1995, at Reel 7393, Frames 0489-0490.

(2) RELATED APPEALS AND INTERFERENCES

The appellant, the appellant's legal representative, and the assignee are not aware of any related prior or pending appeals or interferences or judicial proceedings which will directly affect or be directly affected by or have a bearing on the Board's decision in this appeal.

(3) STATUS OF THE CLAIMS

Claims 24-57 are pending.

Claims 24-57 have been rejected and are the subject of this appeal.

Originally-filed claims 1-23 were canceled, without prejudice, and new claims 24-57 were added in a Preliminary Amendment filed June 5, 2001. Claims 24 and 36-57 were amended in an Amendment dated August 25, 2003.

An Amendment after final rejection filed July 28, 2004 was entered for the purpose of Appeal. See, Advisory Action dated October 13, 2004.

A copy of all the rejected claims 24-57, i.e., the claims involved in the appeal, is attached as a Claims Appendix, pursuant to Rule 41.37(c)(1)(viii).

(4) STATUS OF THE AMENDMENTS

The Amendment filed July 28, 2004 has been entered. See, item 7 of the Advisory Action dated October 13, 2004.

(5) SUMMARY OF CLAIMED SUBJECT MATTER

Independent claim 24 provides an isolated polynucleic acid sequence consisting of 8 or more contiguous nucleotides selected from an HCV subtype 3c genomic sequence, wherein the sequence is capable of hybridizing to HCV type 3c, but not another type or subtype of HCV. Independent claim 24 further provides a complement of the recited sequences.

Independent claim 25 provides an isolated Hepatitis C virus polynucleic acid selected from the nucleotide sequence of SEQ ID NO:147 and at least 8 contiguous nucleotides of a nucleotide sequence having at least one genotype-specific nucleotide from the region spanning positions 1 to 957 of the Core or Core/E1 region of HCV subtype 3c. The complement of a nucleic sequence according to the recited sequences of independent claim 25 are also included in the claim.

Support for independent claim 24 may be found, for example, in original claims 1 and 7, and the following passages of the specification: page 2, lines 29-33; page 3, lines 9 and 17-20; and page 4, lines 28-31.

Support for independent claim 25 may be found, for example, in original claims 1 and 2, and the following passages of the specification: page 14, lines 32-33; page 15, lines 9-11; page 12, lines 23-27; page 62, lines 1-3 and 11-12; and Figures 3 and 4.

Support for the vector of dependent claims 26 and 27 may be found, for example, in originally-filed claim 16, and page 41, line 9 to page 42, line 1 of the specification.

Support for the methods of dependent claims 28-31 may be found, for example, in : originally-filed claim 8 and the following passages of the specification: the 1st full paragraph of page 23, 2nd full paragraph of page 24, 1st full paragraph of page 25, 4th and 5th full paragraphs of page 26, paragraph spanning pages 28-29, and 1st full paragraph of page 29.

Support for the products of dependent claims 32-35 may be found, for example, in originally-filed claim 6 and page 23, line 25 through page 24, line 4 of the specification.

Support for the products of dependent claims 36-39 may be found, for example, in the originally-filed claim 7 and page 24, lines 18-26 of the specification.

Support for the kits of dependent claims 40-49 may be found, for example, in the originally-filed claims 22, 6 and 7; and page 24, last paragraph and the paragraph spanning pages 44-45 of the specification.

Support for the methods of dependent claims 50-57 may be found, for example, in originally-filed claim 8 and the specification in, for example, in the 1st full paragraph of page 23, 2nd full paragraph of page 24, 1st full paragraph of page 25, 4th and 5th full paragraphs of page 26, paragraph spanning pages 28-29, and 1st full paragraph of page 29.

(6) GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The following ground of rejection is presented for review:

(A) Whether the invention of claims 24-57 is supported by an enabling disclosure, as required by 35 U.S.C. § 112, first paragraph.

(B) Whether the invention of claims 24-57 is supported by an adequate written description, as required by 35 U.S.C. § 112, first paragraph.

(7) ARGUMENT

(A) The invention of independent claims 24 and 25, and dependent claims 26-57, is supported by an enabling disclosure, as required by 35 U.S.C. § 112, first paragraph. The Section 112, first paragraph "enablement" rejection of claims 24-57, should be reversed. Consideration of the following in this regard is requested.

The specification teaches on of ordinary skill in the art how to make and use the claimed invention.

Basis for the rejection is stated in the final rejection to be found on page 6 of the Office Action dated April 25, 2003. See, page 2 of the Office Action dated April 1, 2004.

Specifically, the Examiner asserts:

> "The use of long probes will not provide specific hybridization because of the increasing stabilization resulting from the sequences that the various HCV types and subtypes have in common as is evidence by the Figures of the application." See, page 6 of the Office Action dated April 25, 2003.

The Examiner has not provided any technical or scientific support for the assertion that the claimed use of "long" probes will not provide specific hybridization, as claimed.

To the contrary, the appellants have made of record U.S. Patent No. 5,882,852 (attached as evidence (a)), of Bukh et al which was filed August 15, 1994

and evidences the state of the art at the time of the present invention. The Patent Office granted claim 2, for example, of Bukh et al, which provides a method of "determining the major genotype of a hepatitis C virus isolate", with the use of primers consisting of "at least 15 contiguous nucleotides". There is apparently no upper limit of the length of the primer which may be used in Bukh et al.'s claimed method. Bukh et al. further claim products of at least 15 contiguous nucleotides. In granting the claims of Bukh et al., the Patent Office apparently believed the one of ordinary skill in the art could make and use primers (and presumably probes) of unlimited length to amplify HCV genotype-specific sequences.

Moreover, the appellants have made of record U.S. Patent No. 6,548,244 (Maertens et al. - (attached as evidence (b))), which is based on a U.S. application filed in 1993, wherein the Patent Office has granted claims which involve use of genotype-specific HCV sequences where there is no upper or lower limit in the length of the recited sequence.

Finally, the Examiner's reference, Wallace et al., "Oligonucleotide Probes for the Sequencing of Recombinant DNA Libraries", Methods Enzymology, 152, 432-443 (1987) (discussed further below and attached as evidence (c)), includes a section titled "Length" on pages 434-435 which contradict the Examiner regarding the ability of one of ordinary skill in the art to make and use "long" probes for specific hybridization.

Specifically, Wallace et al teach the following:

> "Basically, the length of the oligonucleotide probe determines its hybridization specificity. The longer a sequence, the more likely it is to be unique among the collection of sequences and oligonucleotide is used to probe and the less likely it will bind nonspecifically to other sequences. ... Therefore, oligonucleotide probes should be as long as possible." (Emphasis added.)

The appellants further note that according to Wallace et al, the length not only determines specificity but also duplex stability ("In addition to specificity, oligonucleotide length determines duplex stability." see, lines 11-12 on page 435 of Wallace).

Accordingly, the appellants evidence, as well as the Examiner's citation f Wallace, support the conclusion that one of ordinary skill in the art would have been able to use "long" probes for specific hybridization.

The Examiner has also asserted on page 6 of the Office Action dated April 25, 2003, that one of ordinary skill in the art would not have been able to make and use

> "probes or primers as short as 8 nucleotides for specific hybrdization to HCV DNA sequences. Wallace et al (Methods Enzymol. 152: 432 (1987) teaches that probes shorter than 14 bases long are not suitable for specific hybridization to DNA."

In fact, the passage of Wallace et al. quoted by the Examiner does not exclude the possibility that probes shorter than 14 bases can be found suitable for specific hybridization. Wallace et al specifically state the following:

> "Oligonucleotides have a tendency to bind nonspecifically to noncomplimentary DNA sequences. This is probably due to an unavoidable low degree of homology of short oligonucleotides to other DNA sequences and is a

> particular problem with probes shorter than 14 bases long." Emphasis added, footnotes deleted.

Wallace et al.'s statements regarding "tendencies" are not believed to be as absolute as indicated by the Examiner. Enablement allows for some experimentation. Wallace et al. further specify that the specificity of a probe should first be determined (lines 7-8 on page 434).

Moreover, the appellants have provided evidence in support of the position that one of ordinary skill would believe that probes of less than 14 bases could be made and used without an undue amount of experimentation. Specifically, the appellants have submitted the attached two examples of successful use of probes or primers shorter than 14 nucleotides; Majzoub et al. (1983, PNAS 258, 14061-14064; copy attached as evidence (d)) which successfully utilized primers of 8 bases, and Chan et al. (1979, PNAS 76, 5036-504; copy of abstract attached as evidence (e)) which successfully utilized probes of 10 bases.

The appellants respectfully submit that the claims are supported by an enabling disclosure and that one of ordinary skill would be able to make and use "long" probes as well s probes of less than 14 bases, according to the claimed invention.

The Examiner has further asserted, as a further basis for the "enablement" rejection of dependent method claims 50-57 that the specification allegedly does not teach one of ordinary skill how to amplify specific DNA sequences using only one primer. See, page 6 of the Office Action dated April 25, 2003.

The Board will appreciate however that the methods of dependent claims 50-57 require "at least one" primer and/or one or more probe which are genotype specific. It is not an absolute requirement to use two genotype-specific primers for specific amplification of a DNA sequence. One of ordinary skill in the art will appreciate, for example, that a genotype-specific and a universal primer (see, last full paragraph on page 25 of the specification) could be used. The use of at least one genotype-specific primer and/or one probe is required. In addition, the Examiner appears to view amplification too narrowly, i.e., as PCR. Amplification will be understood by one of ordinary skill in the art however as possibly being obtained with a single primer. The resulting amplification will not be exponential but linear. In a sequencing reaction for example (wherein the target DNA strand is also amplified) a single primer is used.

The claims are submitted to be supported by an enabling disclosure and reversal of the Section 112, first paragraph "enablement", rejection of claims 24-57 is requested.

(B) The invention of independent claims 24 and 25, and dependent claims 26-57, is supported by an adequate written description, as required by 35 U.S.C. § 112, first paragraph. The Section 112, first paragraph "written description" rejection of claims 24-57, should be reversed. Consideration of the following in this regard is requested.

Basis for the rejection is stated in the final rejection to be found on page 6 of the Office Action dated April 25, 2003. See, page 2 of the Office Action dated April 1, 2004.

Specifically, the Examiner asserts:

> "The application does not provide an adequate written description of the claimed invention because no probes with the described desired properties are described. The discussion in the rejection immediately above [i.e., the Section 112, first paragraph "enablement", rejection] is incorporated here." See, page 6 of the Office Action dated April 25, 2003.

As noted above, the claims are literally described and supported by the specification. Moreover, the claimed invention is described in the originally-filed claims, as noted above.

The Federal Circuit has summarized the state of the law with regard to the "written description" requirement of 35 U.S.C. § 112, first paragraph, as follows:

> A fairly uniform standard for determining compliance with the "written description" requirement has been maintained throughout: "Although [the applicant] does not have to describe exactly the subject matter claimed, ... the description must clearly allow persons of ordinary skill in the art to recognize that [he or she] invented what is claimed." In re Gosteli, 872 F.2d 1008, 1012, 10 USPQ2d 1614, 1618 (Fed. Cir. 1989) (citations omitted). "[T]he test for sufficiency of support in a parent application is whether the disclosure of the application relied upon 'reasonably conveys to the artisan that the inventor had possession at that time of the later claimed subject matter.'" Ralston Purina Co. v. Far-Mar-Co, Inc., 772 F.2d 1570, 1575, 227 USPQ 177, 179 (Fed. Cir. 1985) (quoting In re Kaslow, 707 F.2d 1366, 1375, 217 USPQ 1089, 1096 (Fed. Cir. 1983)). Our cases also provide that

> compliance with the "written description" requirement of §112 is a question of fact, to be reviewed under the clearly erroneous standard. Gosteli, 872 F.2d at 1012, 10 USPQ2d at 1618; Utter v. Hiraga, 845 F.2d 993, 998, 6 USPQ2d 1709, 1714 (Fed. Cir. 1988). See, Vas-Cath Inc. v. Mahurkar (CA FC) 19 USPQ2d 1111, 1116

The appellants submit that the specification at least "reasonably conveys to the artisan that the inventor had possession at that time of the later claimed subject matter."

Specifically, a description of independent claim 24 may be found, for example, in original claims 1 and 7, and the following passages of the specification: page 2, lines 29-33; page 3, lines 9 and 17-20; and page 4, lines 28-31;

a description of independent claim 25 may be found, for example, in original claims 1 and 2, and the following passages of the specification: page 14, lines 32-33; page 15, lines 9-11; page 12, lines 23-27; page 62, lines 1-3 and 11-12; and Figures 3 and 4;

a description of the vector of dependent claims 26 and 27 may be found, for example, in originally-filed claim 16, and page 41, line 9 to page 42, line 1 of the specification;

a description of the methods of dependent claims 28-31 may be found, for example, in : originally-filed claim 8 and the following passages of the specification: the 1st full paragraph of page 23, 2nd full paragraph of page 24, 1st full paragraph of page 25, 4th and 5th full paragraphs of page 26, paragraph spanning pages 28-29, and 1st full paragraph of page 29;

a description of the products of dependent claims 32-35 may be found, for example, in originally-filed claim 6 and page 23, line 25 through page 24, line 4 of the specification;

a description of the products of dependent claims 36-39 may be found, for example, in the originally-filed claim 7 and page 24, lines 18-26 of the specification;

a description of the kits of dependent claims 40-49 may be found, for example, in the originally-filed claims 22, 6 and 7; and page 24, last paragraph and the paragraph spanning pages 44-45 of the specification; and

a description of the methods of dependent claims 50-57 may be found, for example, in originally-filed claim 8 and the specification in, for example, in the 1st full paragraph of page 23, 2nd full paragraph of page 24, 1st full paragraph of page 25, 4th and 5th full paragraphs of page 26, paragraph spanning pages 28-29, and 1st full paragraph of page 29.

Moreover, the specification includes a description of probes of the invention as including, for example, 5-50 nucleotides. See, paragraphs 4 and 5 on page 24 of the specification. The specification also includes a description of primers spanning pages 23-24 of the specification. The appellants believe that the Patent Office has, in other applications of the assignees', not distinguished between primers and probes and rejected probes as allegedly being anticipated over art teaching primers. See, Serial No. 09/899,082 being examined by Examiner WHISENANT. (This point was made to the Examiner in the appellants Remarks in the Amendment of August 25, 2003, which the Examiner has not refuted.)

The primers exemplified in the specification (i.e., SEQ ID NOs: 63-82, 124-125 and 141-142) are within the range of nucleotides described.

The pending claims are described throughout the originally-filed claims and specification in a manner that will lead one of ordinary skill to the conclusion that the appellants were in possession of the presently claimed invention at the time the application was filed.

While not believed required to satisfy the requirements of written description, the appellants note that Example 10 of the specification, spanning pages 61-63, further describes the identification of type 3c sequences, both in the Core/E1 region (SEQ ID NO:147) and in the NS5B region (SEQ ID NO:149).

Finally, for completeness, the appellants submit that the Examiner's reference to arguments made in support of a Section 112, first paragraph "enablement" rejection should not be sufficient to support a Section 112, first paragraph "written description" rejection, as the Federal Circuit is believed to have described "enablement" and "written description" as separate requirements. Id. The above comments and cited passages of the specification are believed to be sufficient to demonstrate that the specification adequately describes the claimed invention.

The claims are submitted to be supported by an adequate written description and reversal of the Section 112, first paragraph "written description", rejection of claims 24-57 is requested.

The claims are submitted to be in condition for allowance and Reversal of the Final Rejection is requested.

Respectfully submitted,

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(8) CLAIMS APPENDIX

24. An isolated polynucleic acid sequence consisting of 8 or more contiguous nucleotides selected from an HCV subtype 3c genomic sequence, wherein said polynucleic acid sequence is capable of hybridizing to HCV type 3c, but not another type or subtype of HCV; or

the complement of said polynucleic acid, wherein said polynucleic acid contains at least one genotype 3c-specific nucleotide.

25. An isolated Hepatitis C virus polynucleic acid selected from the group consisting of:

(i) the nucleotide sequence of SEQ ID NO:147,

(ii) at least 8 contiguous nucleotides of a nucleotide sequence having at least one genotype-specific nucleotide from the region spanning positions 1 to 957 of the Core or Core/E1 region of HCV subtype 3c, and,

(iii) the complement of the nucleotide sequence of (i) or (ii).

26. A recombinant vector comprising a vector sequence and a prokaryotic, eukaryotic or viral promotor sequence operably linked to a polynucleic acid sequence of claim 24.

27. A recombinant vector comprising a vector sequence and a prokaryotic, eukaryotic or viral promotor sequence operably linked to a polynucleic acid sequence of claim 25.

28. A method of detecting or screening for one or more HCV genotypes present in a biological sample, comprising the following steps:

(i) providing a sample nucleic acid,

(ii) determining the presence of a polynucleic acid sequence according to claim 24, by means of a sequencing reaction, and,

(iii) inferring from the presence of one or more of these HCV polynucleic acid sequences of step (ii) the genotype(s) present in said sample.

29. A method of detecting or screening for one or more HCV genotypes present in a biological sample, comprising the following steps:

(i) providing a sample nucleic acid,

(ii) determining the presence of a polynucleic acid sequence according to claim 25, by means of a sequencing reaction, and,

(iii) inferring from the presence of one or more of these HCV polynucleic acid sequences of step (ii) the genotype(s) present in said sample.

30. A method of detecting or screening for one or more HCV genotypes

present in a biological sample, comprising the following steps:

(i) providing a sample nucleic acid,

(ii) specifically amplifying a polynucleic acid sequence according to claim 24, and,

(iii) inferring from the presence of one or more amplified HCV polynucleic acid sequences of step (ii) the genotype(s) present in said sample.

31. A method of detecting or screening for one or more HCV genotypes present in a biological sample, comprising the following steps:

(i) providing a sample nucleic acid,

(ii) specifically amplifying a polynucleic acid sequence according to claim 25, and,

(iii) inferring from the presence of one or more amplified HCV polynucleic acid sequences of step (ii) the genotype(s) present in said sample.

32. An isolated HCV polynucleic acid according to claim 24, wherein said polynucleic acid is capable of acting as a primer for HCV type- or subtype-specific amplification, and wherein said polynucleic acid consists of up to 50 contiguous nucleotides selected from said HCV subtype 3c genomic region.

33. An isolated HCV polynucleic acid according to claim 25, wherein said polynucleic acid is capable of acting as a primer for HCV type- or subtype-specific amplification, and wherein said polynucleic acid consists of up to 50 contiguous nucleotides selected from said HCV subtype 3c genomic region.

34. An isolated HCV polynucleic acid according to claim 24, wherein said polynucleic acid is capable of acting as a primer of a HCV subtype 3c nucleic acid sequence, and wherein said polynucleic acid consists of up to 50 contiguous nucleotides selected from said HCV subtype 3c genomic region.

35. An isolated HCV polynucleic acid according to claim 25, wherein said polynucleic acid is capable of acting as a primer of a HCV subtype 3c nucleic acid sequence, and wherein said polynucleic acid consists of up to 50 contiguous nucleotides selected from said HCV subtype 3c genomic region.

36. An isolated HCV polynucleic acid according to claim 24, wherein said polynucleic acid is capable of acting as a probe for specific hybridization to a HCV type or subtype-specific, and wherein said polynucleic acid consists of up to 50 contiguous nucleotides selected from said HCV subtype 3c genomic region.

37. An isolated HCV polynucleic acid according to claim 25, wherein said polynucleic acid is capable of acting as a probe for specific hybridization to a HCV type or subtype-specific, and wherein said polynucleic acid consists of up to 50 contiguous nucleotides selected from said HCV subtype 3c genomic region.

38. An isolated HCV polynucleic acid according to claim 24, wherein said polynucleic acid is capable of acting as a probe for specific hybridization to a HCV subtype 3c nucleic acid sequence, and wherein said polynucleic acid consists of up to 50 contiguous nucleotides selected from said HCV subtype 3c genomic region.

39. An isolated HCV polynucleic acid according to claim 25, wherein said polynucleic acid is capable of acting as a probe for specific hybridization to a HCV subtype 3c nucleic acid sequence, and wherein said polynucleic acid consists of up to 50 contiguous nucleotides selected from said HCV subtype 3c genomic region.

40. A kit for determining the presence of HCV genotypes comprising a solid support and a polynucleic acid sequence according to claim 24.

41. A kit for determining the presence of HCV genotypes comprising a solid support and a polynucleic acid sequence according to claim 25.

42. A kit for determining the presence of HCV genotypes comprising a solid support and a primer according to claim 32.

43. A kit for determining the presence of HCV genotypes comprising a solid support and a primer according to claim 33.

44. A kit for determining the presence of HCV genotypes comprising a solid support and a primer according to claim 34.

45. A kit for determining the presence of HCV genotypes comprising a solid support and a primer according to claim 35.

46. A kit for determining the presence of HCV genotypes comprising a solid support and a probe according to claim 36.

47. A kit for determining the presence of HCV genotypes comprising a solid support and a probe according to claim 37.

48. A kit for determining the presence of HCV genotypes comprising a solid support and a probe according to claim 38.

49. A kit for determining the presence of HCV genotypes comprising a solid support and a probe according to claim 39.

50. A method for determining the presence of HCV genotypes present in a biological sample comprising the steps of:

(i) providing a sample nucleic acid,

(ii) amplifying the nucleic acid with at least one primer according to claim 32,

(iii) detecting the amplified nucleic acids,

(iv) inferring the presence of one or more genotypes of HCV present from the observed pattern of amplified fragments.

51. A method for determining the presence of HCV genotypes present in a biological sample comprising the steps of:

(i) providing a sample nucleic acid,

(ii) amplifying the nucleic acid with at least one primer according to claim 33,

(iii) detecting the amplified nucleic acids,

(iv) inferring the presence of one or more genotypes of HCV present from the observed pattern of amplified fragments.

52. A method for determining the presence of HCV genotypes present in a biological sample comprising the steps of:

(i) providing a sample nucleic acid,

(ii) amplifying the nucleic acid with at least one primer according to claim 34,

(iii) detecting the amplified nucleic acids,

(iv) inferring the presence of one or more genotypes of HCV present from the observed pattern of amplified fragments.

53. A method for determining the presence of HCV genotypes present in a biological sample comprising the steps of:

(i) providing a sample nucleic acid,

(ii) amplifying the nucleic acid with at least one primer according to claim 35,

(iii) detecting the amplified nucleic acids,

(iv) inferring the presence of one or more genotypes of HCV present from the observed pattern of amplified fragments.

54. A method for determining the presence of HCV genotypes present in a

biological sample comprising the steps of:

(i) providing a sample nucleic acid,

(ii) optionally amplifying the nucleic acid with at least one primer,

(iii) hybridizing the nucleic acids of the biological sample with one or more probes according to claim 36, with said probes being optionally attached to a solid substrate,

(iv) optionally washing,

(v) detecting the hybrids formed,

(vi) inferring the presence of one or more genotypes of HCV present from the observed hybridization pattern.

55. A method for determining the presence of HCV genotypes present in a biological sample comprising the steps of:

(i) providing a sample nucleic acid,

(ii) optionally amplifying the nucleic acid with at least one primer,

(iii) hybridizing the nucleic acids of the biological sample with one or more probes according to claim 37, with said probes being optionally attached to a solid substrate,

(iv) optionally washing,

(v) detecting the hybrids formed,

(vi) inferring the presence of one or more genotypes of HCV present from the

observed hybridization pattern.

56. A method for determining the presence of HCV genotypes present in a biological sample comprising the steps of:

(i) providing a sample nucleic acid,

(ii) optionally amplifying the nucleic acid with at least one primer,

(iii) hybridizing the nucleic acids of the biological sample with one or more probes according to claim 38, with said probes being optionally attached to a solid substrate,

(iv) optionally washing,

(v) detecting the hybrids formed,

(vi) inferring the presence of one or more genotypes of HCV present from the observed hybridization pattern.

57. A method for determining the presence of HCV genotypes present in a biological sample comprising the steps of:

(i) providing a sample nucleic acid,

(ii) optionally amplifying the nucleic acid with at least one primer,

(iii) hybridizing the nucleic acids of the biological sample with one or more probes according to claim 39, with said probes being optionally attached to a solid substrate,

(iv) optionally washing,

(v) detecting the hybrids formed,

(vi) inferring the presence of one or more genotypes of HCV present from the observed hybridization pattern.

(9) EVIDENCE APPENDIX

Attached:

(a) U.S. Patent No. 5,882,852, Bukh et al. issued May 7, 1996.

Cited by appellants in Information Disclosure Statement filed August 25, 2003 and indicated as considered by Examiner with PTO 1449 Form signed and dated March 31, 2004.

(b) U.S. Patent No. 6,548,244, Maertens et al., issued April 15, 2003.

Cited by appellants in Information Disclosure Statement filed August 25, 2003 and indicated as considered by Examiner with PTO 1449 Form signed and dated March 31, 2004.

(c) Wallace et al., "Oligonucleotide Probes for the Sequencing of Recombinant DNA Libraries", Methods Enzymology, 152, 432-443 (1987)

Cited by the appellants in Information Disclosure Statement filed with the application and indicated as considered by Examiner with PTO 1449 Form signed and dated April 15, 2003.

(d) Majzoub et al. (1983, PNAS 258, 14061-14064.

Cited by the appellants in Information Disclosure Statement filed August 25, 2003 and indicated as considered by Examiner with PTO 1449 Form signed and dated March 31, 2004.

(e) Chan et al. (1979, PNAS 76, 5036-504.

Cited by the appellants in Information Disclosure Statement filed August 25, 2003 and indicated as considered by Examiner with PTO 1449 Form signed and dated March 31, 2004.

(9) RELATED PROCEEDINGS APPENDIX

Attached:

NONE

#3047

gives a significant advantage regarding the sensitivity of screening. Since only a few different clones exist in the library, a large fraction of its complexity can be screened by preparing DNA from a manageably small number of clones, perhaps only a few dozen. This DNA can be radiolabeled and used to probe RNA gel or dot blots containing samples from various sources. Extremely rare RNAs can be easily detected by such methods. Clones can also be screened by sequence determination, hybrid-selected or arrested translation, or genomic blot analysis.

Appendix: Preparation of Carrier DNA

1. Dissolve 1 g DNA (e.g., salmon testes DNA, Sigma D-1626) in 50 ml 0.1 *N* NaOH. Add 1.5 ml concentrated HCl, and mix quickly. The DNA will precipitate immediately, and should not be stirred more than a few seconds to prevent formation or a large aggregate.
2. Incubate at room temperature for 20 min to partially depurinate the DNA. Add 2 ml 10 *N* NaOH (OH^- concentration to 0.1 *N*) and stir until the DNA redissolves completely.
3. Incubate at 65° for 30 min. This hydrolyzes the DNA to a broad size distribution, from 250 to around 1000 nucleotides. Cool to room temperature, and add SDS to 0.5%.
4. Extract twice with phenol–chloroform (1 : 1).
5. Add 7 ml 3 *M* sodium acetate (pH 4.5), mix, and then add 60 ml 2-propanol.
6. Chill 15 min in dry ice to precipitate DNA.
7. Centrifuge to recover precipitate, which is then washed with 70% ethanol, decanted (do not dry the DNA), and finally dissolved in 50 ml 25 m*M* NaOH.
8. Store at 5° in the 25 m*M* NaOH to prevent renaturation. Do not freeze DNA in NaOH.

[47] Oligonucleotide Probes for the Screening of Recombinant DNA Libraries

By R. BRUCE WALLACE and C. GARRETT MIYADA

#3047

There are several situations in which the logical screening strategy is to use a synthetic oligonucleotide as a hybridization probe. When a portion of the amino acid sequence of the protein is known, an oligonucleotide probe can be designed based on this information. This idea was

Methods Enzymol. 152: 432 (1987)

(127)

#3041

first proposed[1,2] when it was determined that oligonucleotides hybridize to their complementary sequence with a high degree of specificity. Under appropriate conditions only duplexes will form in which all of the nucleotides are base paired, while mismatched duplexes will not. Thus, it is possible to design an oligonucleotide probe that is a mixture of all possible coding sequences for a given amino acid sequence. Only one of the oligonucleotides in the mixture is complementary to the coding region of the protein. All of the other sequences in the mixture are only capable of forming mismatched duplexes with this region.

Oligonucleotides of unique sequence are also useful for screening recombinant DNA libraries. The hybridization specificity of oligonucleotide probes allows one to use unique sequence probes to screen for genomic clones or cDNAs encoding a specific member of a multigene family,[3] to screen for a new allele when the sequence of one allele is known, to screen for a specific region of a gene, to screen for specific mutants created by site-directed mutagenesis,[4,5] or to screen libraries with probes whose sequence represents a consensus coding sequence.[6]

This chapter deals with procedures for the use of oligonucleotides as hybridization probes including probe design, labeling, and hybridization to colonies, phage plaques, DNA, and RNA. It does not deal with oligonucleotide synthesis.

Screening Strategy

Oligonucleotides have several unique properties which make them suitable as probes for screening recombinant DNA libraries. The major unique property is the hybridization specificity discussed above. There are also some limitations of oligonucleotides which should be considered when designing experiments which use these probes. The major limitation when using mixed oligonucleotide probes is that often there is no obvious positive control to use. Oligonucleotides have a tendency to bind nonspe-

[1] R. B. Wallace, J. Shaffer, R. F. Murphy, J. Bonner, T. Hirose, and K. Itakura, *Nucleic Acids Res.* **6**, 3543 (1979).
[2] R. B. Wallace, M. J. Johnson, T. Hirose, T. Miyake, E. H. Kawashima, and K. Itakura, *Nucleic Acids Res.* **9**, 879 (1981).
[3] D. H. Schulze, L. R. Pease, Y. Obata, S. G. Nathenson, A. A. Reyes, S. Ikuta, and R. B. Wallace, *Mol. Cell. Biol.* **3**, 750 (1983).
[4] R. B. Wallace, P. F. Johnson, S. Tanaka, M. Schold, K. Itakura, and J. Abelson, *Science* **209**, 1396 (1980).
[5] R. B. Wallace, M. Schold, M. J. Johnson, P. Dembek, and K. Itakura, *Nucleic Acid Res.* **9**, 3647 (1981).
[6] R. Lathe, *J. Mol. Biol.* **183**, 1 (1985).

cifically to noncomplementary DNA sequences. This is probably due to an unavoidable low degree of homology of short oligonucleotides to other DNA sequences[6] and is a particular problem with probes shorter than 14 bases long. Thus, one is often faced with the problem of falsely identifying clones as positive due to the lack of an appropriate control. Negative controls should always be used. When possible, the specificity of the probe should be determined, for example by using it in a Northern blot experiment (see Ito *et al.*[7] for example, and this volume [61]).

When using mixed oligonucleotides as probes for cDNA clones, it is best to choose the longest probe possible, preferably 20 or longer, as the primary probe. If one has additional protein sequence information, it is desirable to synthesize a second probe to another region. This probe can be used to rescreen positive clones obtained with the first probe, either in a colony screening experiment, or by Southern blotting (see this volume [45, 61]) or dried gel hybridization with DNA obtained from miniplasmid or miniphage preparations. Clones positive with the two independent probes are very likely to be the desired clone.

Probe Design

In the design of oligonucleotide probes for any application, several aspects of the DNA sequence must be considered. These include oligonucleotide length, G + C content, self-complementarity, and complexity (in the case of mixed oligonucleotide probes). These aspects shall be considered separately.

Length

Basically, the length of the oligonucleotide probe determines its hybridization specificity. The longer a sequence, the more likely it is to be unique among the collection of sequences and oligonucleotide is used to probe and the less likely it will bind nonspecifically to other sequences. The length of the oligonucleotide is limited more by need and appropriate information than by synthetic considerations, since it is possible to synthesize oligonucleotides in excess of 100 nucleotides in length on most of the modern DNA synthesizers.

For a genome whose nucleotides are distributed randomly, the expected frequency of occurrence (a) of an oligonucleotide sequence is

$$a = (g/2)^{G+C} \times [(1 - g)/2]^{A+T} \tag{1}$$

[7] H. Ito, S. Yamamoto, S. Kuroda, H. Sakamoto, J. Kajihara, T. Kiyota, H. Hayashi, M. Kato, and M. Seko, *DNA* **5**, 149 (1986).

where g is the fractional G + C content of the genome, and G, C, A, and T are the number of guanines, cytosines, adenines, and thymines, respectively, in the oligomeric sequence.[8] For a genome of size N (in nucleotide pairs) the expected total number n of oligonucleotide-complementary sites is

$$n = 2Na \tag{2}$$

since the oligonucleotide could be complementary to either DNA strand. As stated by Nei and Li,[8] n follows a Poisson distribution with mean of $2Na$.

In addition to specificity, oligonucleotide length determines duplex stability.[1] This is particularly important for oligonucleotides 11 bases long or shorter. While these sequences might be expected to be unique on statistical grounds, they have been found to give unacceptable results as probes for cloned DNAs.[9] Therefore, oligonucleotide probes should be made as long as possible.

G + C Content

The G + C content of an oligonucleotide is also important in duplex stability.[10] While no detailed thermodynamic study has been done, Suggs *et al.*[10] have determined an empirical effect of oligonucleotide G + C content on duplex stability. By comparing the dissociation of several oligonucleotide DNA duplexes as a function of temperature, a parameter T_d (the temperature at which half of the duplex is dissociated) was determined.[1] When the effect of G + C content was taken into account, an empirical relationship was derived

$$T_d = 2^\circ \text{ (number of A + T residues)} + 4^\circ \text{ (number of G + C residues)} \tag{3}$$

for duplexes 11–23 bases long in 1 M Na^+. This relationship is only valid within these ranges of length and is meant to serve as a guide. Smith[11] has presented a more detailed analysis of the effect of length on duplex stability. The above relationship is useful for estimating the effects of length

[8] M. Nei and W.-H. Li, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5269 (1979).

[9] S. V. Suggs, R. B. Wallace, T. Hirose, E. H. Kawashima, and K. Itakura, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6613 (1981).

[10] S. V. Suggs, T. Hirose, T. Miyake, E. H. Kawashima, M. J. Johnson, K. Itakura, and R. B. Wallace, *in* "Developmental Biology Using Purified Genes" (D. D. Brown, ed.), p. 683. Academic Press, New York, 1981.

[11] M. Smith, *in* "Methods of RNA and DNA Sequencing" (S. M. Weissman, ed.), p. 23. Praeger, New York, 1983.

and G + C content on duplex stability, as well as for determining an appropriate hybridization temperature (see below).

In addition to effects on the duplex stability, G content of oligonucleotides has an effect on the DNA synthesis itself, particularly on purification of the oligonucleotide. G-rich oligonucleotides are notoriously difficult to purify. Although purification schemes have been devised,[12] it is best to avoid the problem when possible. This is often achieved by simply synthesizing the complementary sequence which would serve the same purpose for many applications. Alternatively, synthesis of an oligonucleotide complementary to a different region is required.

Self-Complementarity

Self-complementarity of oligonucleotides, like G content, creates problems for oligonucleotide purification.[12] Unlike G richness, it is obviously not possible to avoid self-complementarity by synthesis of the complementary sequence. It has not been determined what effect, if any, oligonucleotide self-complementarity would have on the efficiency of hybridization to a complementary DNA or RNA sequence. However, it is possible that self-complementarity would affect 5′ labeling and should be avoided whenever possible.

Complexity

When synthesizing a mixed-sequence probe, the complexity (number of different oligonucleotides in the mixture) must be taken into account. Increases in the mixture complexity result in two effects, a decreased hybridization specificity and a decreased abundance of the single correct probe in the mixture. No detailed studies have been done to determine the maximal acceptable complexity of the mixed probes. Wherever possible, the complexity should be minimized. Single mixtures as complex as 384 different sequences have been used successfully.[13] However, complex probe regions can be handled by the synthesis of two or more pools of mixtures for the same region. A recent good example of this approach is the cloning of the cDNA for rabbit tumor necrosis factor (TNF) by Ito *et al.*[7] In this study a region of protein sequence was encoded by 128 different possible nucleotide sequences. Five pools were synthesized, and each pool hybridized to a Northern blot of RNA isolated from rabbit macrophages which had been stimulated to produce TNF. The pool which gave

[12] M. D. Edge, A. R. Greene, G. R. Heathcliffe, P. A. Meacok, W. Schuch, D. B. Scanlon, T. C. Atkinson, C. R. Newton, and A. F. Markham, *Nature (London)* **292**, 756 (1981).

[13] A. S. Whitehead, G. Goldberger, D. E. Woods, A. F. Markham, and H. R. Colten, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5387 (1983).

the strongest hybridization signal was used as the hybridization probe in the subsequent cloning experiment. Clearly, antisense oligomers must be used for analysis of mRNA.

Hybridization Conditions

The G + C content and the length of an oligonucleotide affect the thermal stability of the duplex formed (above). The parameter T_d is also useful for determining the appropriate hybridization temperature. Suggs *et al.*[10] have suggested that hybridization should be done at 2–5° below the calculated T_d such that duplexes with one or more mismatched base pairs will not form.

One aspect of oligonucleotide hybridization that is little appreciated is the kinetics of the reaction. Since synthetic oligonucleoides are of low sequence complexity and are available in large amounts, hybridization rates are much higher than those normally encountered in the molecular biology laboratory. In the most typical application of oligonucleotide probes, a labeled oligonucleotide is hybridized with DNA immobilized on a solid matrix. The oligonucleotide is present in vast excess with respect to the immobilized DNA sequences. Hybridization kinetics are essentially first order with respect to oligonucleotide concentration. The rate constant (k) as a function of length and complexity of the probe has been described by Wetmur and Davidson[14] as

$$k = 3 \times 10^5 L^{0.5} N^{-1} \quad \text{(liters per mole of nucleotides per second)} \qquad (4)$$

where L is length and N is complexity of the probe in nucleotides for hybridizations done in 1 M Na^+. Since the reaction is first order, then

$$t_{1/2} = (\ln 2)/kC \qquad (5)$$

where C is the concentration of probe in moles of nucleotides per liter. As an example, for a 15-base long oligonucleotide hybridized at 0.01 μg/ml, the hybridization is half complete in about 4 min. Wallace *et al.*[1] have shown, however, that the actual rate of hybridization is about 3–4 times slower than the calculated rate. Thus in the above example, the half-time of hybridization is 12–16 min. (See also this volume [43] for a more general discussion. The rate constant k is defined in Eq. (5) under "standard" conditions.)

Reagents

Denhardt's solution: 0.02% bovine serum albumin, 0.02% poly(vinylpyrrolidone), 0.02% Ficoll

[14] J. G. Wetmur and N. Davidson, *J. Mol. Biol.* **31**, 349 (1968).

Kinase buffer: 67 mM Tris–HCl (pH 8.3), 10 mM $MgCl_2$, 10 mM dithiothreitol

NET: 0.15 M NaCl, 15 mM Tris–HCl (pH 8.3), 1 mM EDTA

SSC: 0.15 M NaCl, 0.015 M sodium citrate

SSPE: 180 mM NaCl, 10 mM sodium phosphate, 1 mM Na_2EDTA (pH 8)

TBE running buffer: 89 mM Tris base, 89 mM boric acid, 2 mM EDTA

TE: 10 mM Tris–HCl (pH 8.0), 1 mM EDTA

Procedures

Determination of Oligonucleotide Concentration

Like other nucleic acids, oligonucleotides are measured spectrophotometrically. However, since the base composition of different synthetic sequences can vary widely, it is necessary to calculate the molar extinction coefficient for the particular sequence to permit determination of the concentration. The molar extinction coefficient at 260 nm (pH 8.0) is calculated by summing the contribution of each base: G, 12,010; A, 15,200; T, 8400; and C, 7050. The concentration of a solution of the oligonucleotide can then be determined by measurement of the absorbance of the solution at 260 nm. Solutions of oligonucleotides should be made in TE and stored frozen.

Labeling of Oligonucleotides

Oligonucleotides synthesized by the phosphotriester or the phosphoramidite approaches contain a free 5′-OH. There are two general methods of radiolabeling these molecules, labeling by phosphorylation of the 5′-OH with [^{32}P]phosphate and by primer extension.[15] The later approach will not be discussed here because it is not generally useful for mixed oligonucleotide probes.

The oligonucleotide which has a 5′-OH is labeled by transfer of the [^{32}P]phosphate from [γ-^{32}P]ATP using T4 polynucleotide kinase. A typical kinase reaction contains in 10 μl of kinase buffer: 18.5 pmol oligonucleotide, 30 pmol [γ-^{32}P]ATP (>7000 Ci/mmol, New England Nuclear), 5–6 units of polynucleotide kinase. The reaction is incubated at 37° for 30 min. An equal volume of deionized 98% formamide containing 0.15% bromphenol blue and 0.15% xylene cyanole is added; the sample is heated 5 min at 95°, and then subjected to electrophoresis on a 14.5% acrylamide, 0.5% bisacrylamide gel (15 cm × 30 cm × 1 mm) containing 7 M urea in

[15] A. B. Studencki and R. B. Wallace, *DNA* 3, 7 (1984).

TBE buffer. Electrophoresis is done at 27.5 mA until the bromphenol blue reaches the gel bottom. The labeled oligonucleotide is located by autoradiography (see this volume [7, 8]), the radioactive band excised, and the oligonucleotide eluted by soaking the gel slice in two 300-μl changes of TE over a period of at least 12 hr at 37°. This procedure removes the excess [γ-^{32}P]ATP and separates the phosphorylated oligonucleotide from any remaining unlabeled probe.

If the oligonucleotide has been previously purified by gel or HPLC, the unincorporated [γ-^{32}P]ATP may be removed by chromatography on DE-52 cellulose (Whatman). After the kinase reaction, the labeled oligonucleotide is diluted 10-fold in TE. The sample is then applied to a small column of DE-52 (bed volume, approximately 0.2 ml), previously equilibrated with TE. The column is then washed with 5 bed volumes of TE followed by 5 bed volumes of 0.2 M NaCl in TE, which removes the unincorporated label. The labeled oligonucleotide is then eluted with 0.5 ml of 1 M NaCl in TE and used directly in hybridization experiments. Kinased oligonucleotides prepared by either method may be used for at least 1 week when stored at −20°.

Colony Screening

A typical hybridization procedure for screening filter replicas from a recombinant DNA library is shown in Table I. Colonies containing recombinant plasmids are transferred to filters as described in this volume [44] or they can be transferred to Whatman 540 filter circles and prepared for hybridization as described by Gergen *et al.*[16] In the case of nitrocellulose filters a prehybridization step is included as described below for plaque screening. In the case of the cellulose filters, the filters are first rinsed in a solution containing 6× NET, 5× Denhardt's solution, and 0.1% sodium dodecyl sulfate (SDS). The filters are hybridized in the above solution containing 1 × 10^6 cpm/ml labeled oligonucleotide (or up to 1 × 10^7 cpm/ml for mixed probes) at the appropriate temperature, T_d − 5° for unique sequence probes and $T_{d\min}$ − 2° for mixed probes, for 2 hr. The filters are then washed four times for 5 min each in 6× SSC at 30–40° below the hybridization temperature. The filters are then exposed to Kodak XAR-5 film with two Cronex Lightning Plus intensifier screens for 1–4 hr at −70°. The filters can then be washed in 6× SSC for 1 min at the hybridization temperature and reexposed to X-ray film. By comparing the two films the consequence of the higher temperature wash can be assessed and any positive colonies identified. Additional washes in 6× SSC are used as needed to control background. All stringent washes (at T_d − 5°) are done for short times (1–5 min).

[16] J. P. Gergen, R. H. Stern, and P. C. Wensink, *Nucleic Acids Res.* 7, 2115 (1979).

TABLE I
CONDITIONS FOR OLIGONUCLEOTIDE HYBRIDIZATION[a]

Step	Conditions
Prehybridization (for nitrocellulose)	
Solution	6× NET, 0.1% SDS, 5× Denhardt's solution, 100 μg/ml *E. coli* DNA
Incubation	65° for 5 hr
Hybridization	
Solution	6× NET, 0.1% SDS, 5× Denhardt's solution, 1–10 ng/ml ^{32}P-labeled oligonucleotide
Incubation	$T_d - 5°$ ($T_{dmin} - 2°$ for mixed probes) for 2 hr
Washing	
Solution	6× SSC, 0.1% SDS
Initial wash	Four changes at 4° for 5 min each
Initial X-ray film exposure	1–24 hr (typically)
Additional washes	As needed to control background
Final wash	One change at hybridization temperature for 1–2 min
Final X-ray film exposure	Same time as initial exposure

[a] A typical procedure for hybridizing immobilized DNA with labeled oligonucleotide probes.

Plaque Screening

A prehybridization step is beneficial in the screening of recombinant phage by the method of Benton and Davis[17] with oligonucleotide probes. The filters are prehybridized in a solution containing 6× NET, 5× Denhardt's solution, 0.1% SDS, and 100 μg/ml sonicated, denatured *E. coli* DNA at 65° for 4–16 hr. The prehybridization solution is then removed and the filters are hybridized and washed as described in the colony screening section. It is necessary to expose the filters to X-ray film about 10 times longer than for the colony screening due to the lower signals obtained with phage plaques.

Other Oligonucleotide Hybridization Techniques

Hybridization to DNA in Dried Agarose Gels (Unblots)

In our hands, oligonucleotide probes produce a hybridization signal with a genomic restriction digest (in which fragments are >1.5 kb) approximately five times stronger when hybridized directly in the agarose

[17] W. D. Benton and R. W. Davis, *Science* **196,** 180 (1977).

gel matrix than when the same digest has been transferred to nitrocellulose or similar hybridization membrane.

Preparation of DNA Samples for Dried Gel Hybridization.[18] After electrophoresis, gels are stained with ethidium bromide and then photographed. The DNA samples are denatured by soaking the gel in 0.5 *N* NaOH, 150 m*M* NaCl for 30 min at room temperature with gentle shaking. The gel is then neutralized by soaking the gel in 500 m*M* Tris–HCl (pH 8), 150 m*M* NaCl for 30 min at 4° with occasional shaking. The gel is then placed on two sheets of Whatman 3 MM paper. The gel is trimmed at this point to remove any unused lanes. The gel (still on the Whatman paper) is covered with plastic wrap, placed in a gel dryer (Bio-Rad), covered with only the neoprene rubber sheet, and dried with only the vacuum until the gel is nearly flat (approximately 30 min). Then the gel dryer heater is turned on to 60° and the gel is dried an additional 0.5–1 hr. The vacuum is then released. At this point the gel has been reduced to a thin membrane on the Whatman paper. The gel is now ready for hybridization or it can be stored at room temperature indefinitely on the paper after wrapping it in plastic wrap.

Dried Gel Hybridizations.[19] The following conditions apply to the hybridization of an oligonucleotide probe to a single-copy genomic sequence and allow the discrimination between two genes that differ by a single nucleotide. The dried gel is removed from its paper backing by soaking it in a shallow dish of distilled water. Although the gel is no longer stuck to the paper, the gel is supported by the paper to facilitate its handling. After the gel is blotted to remove excess water, the gel and supporting paper are placed in a Seal-a-Meal (Dazey) plastic bag. The gel is pressed against the Seal-a-Meal bag, which leads to the preferential adherence of the gel to the plastic and allows the paper backing to be removed. Prehybridization is not necessary. The dried gel is hybridized in a solution that contains 5× SSPE, 0.1% SDS, 10 μg/ml sonicated, denatured *E. coli* or salmon sperm DNA, and 2 × 10^6 cpm/ml of labeled oligonucleotide. The hybridization volume for a 10 × 14 cm dried gel is 6 ml. The gel is hybridized at $T_d - 5°$ or 60° whichever is lower for 16 hr. After the hybridization period, the gel is washed with 6× SSC first at room temperature then at a stringent temperature. Usually two 15-min washes at room temperature are done followed by a 4-hr wash at room temperature. The stringent wash is done in 6× SSC at $T_d - 5°$ for 1–3 min. Gels can be reused for additional hybridizations by merely subjecting them to the alkali denaturation and neutralization as described in the section on drying the gels.

[18] S. G. S. Tsao, C. F. Brunk, and R. E. Perlman, *Anal. Biochem.* **131,** 365 (1983).
[19] C. G. Miyada, C. Klofelt, A. A. Reyes, E. McLaughlin-Taylor, and R. B. Wallace, *Proc. Natl. Acad. Sci. U.S.A.* **82,** 2890 (1985).

After the appropriate washes, the hybridized gel is wrapped with plastic wrap and exposed to Kodak XAR-5 X-ray film between two Cronex Lightning Plus intensifying screens at −70° (this volume [7]).

Hybridization to RNA in Dried Agarose Gels (Unblots)

RNA can also be hybridized in a dried gel. After electrophoresis of RNA in formaldehyde-containing gels the gel is washed for 30 min in 0.1 *M* Tris–HCl (pH 7.5), dried, and hybridized as described for the DNA dried gel. RNA molecules as small as 9 S (globin mRNA) appear to be retained by this procedure. Hybridization conditions are a described for DNA hybridization in dried gels.

Hybridization to DNA and RNA Bound to Membranes

Oligonucleotide hybridizations to restriction fragments less than 1.5 kb in size require the transfer of the DNA to a hybridization membrane since restriction fragments of this size tend to be lost from the agarose gel matrix during the hybridization and subsequent washes. Acid depurination (nicking)[20] of the DNA, which facilitates its transfer out of the gel, should be avoided since oligonucleotides do not hybridize efficiently to DNA treated in this manner. Conditions for electrophoresis of DNA and RNA and transfer to membranes are described in this volume [8, 61]. Blots are prehybridized, hybridized, and washed using conditions described in Table I. (See also this volume [43, 45, 61].)

Acknowledgments

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[20] G. M. Wahl, M. Stern, and G. R. Stark, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3683 (1979).

[48] Gene Cloning Based on Long Oligonucleotide Probes

By WILLIAM I. WOOD

The most commonly used technique for gene cloning has been to utilize oligonucleotide probes based on protein sequence data. Of course this approach requires characterized and purified protein so that at least a portion of amino acid sequence can be determined and used to infer the corresponding DNA sequence. Based on the amino acid sequence information, either short or long oligonucleotide probes can be synthesized chemically.

Short probes are typically 11–20 bases in length and are pools of 8–32 (or more) sequences including all of the possible codon choices for each amino acid. There are three disadvantages of short probes. (1) They can generally only be used in regions of low codon redundancy; otherwise the pool size becomes unmanageable. (2) The amino acid sequence must be correct. A single mismatch is generally sufficient to prevent hybridization of the probe. (3) Only probes of 17 or longer can be used to screen high-complexity libraries (e.g., a human genomic library). This is because the complexity of the mammalian genome is such that an exact match of any 16-base sequence would be expected at random. When a pool of sequences is used, the number of false positives can be a problem. In some cases this difficulty can be overcome by using two nearby short probes. The advantage of short probes is that if the protein sequence data are correct, the probe should hybridize faithfully as all the codon choices are covered. Also, the exact hybridization conditions used need not be determined empirically when tetramethylammonium chloride is used[1] (see also this volume [49]).

Long probes on the other hand are typically 30–100 nucleotides long and are a single sequence based on a best guess for each codon. The long probe approach was first used to screen for three different genes: bovine tryspin inhibitor,[2] human insulin-like growth factor I,[3] and human factor IX.[4] There are three advantages of long probes. (1) Any stretch of amino acid sequence 10 or longer can be used; regions of low redundancy while

[1] W. I. Wood, J. Gitschier, L. A. Lasky, and R. M. Lawn, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 1585 (1985).
[2] S. Anderson and I. B. Kingston, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 6838 (1983).
[3] A. Ullrich, C. H. Berman, T. J. Dull, A. Gray, and J. M. Lee, *EMBO J.* **3**, 361 (1984).
[4] M. Jaye, H. de la Salle, F. Schamber, A. Ballard, V. Kohij, A. Findeli, P. Tolstoshev, and J. P. Lecocq, *Nucleic Acids Res.* **11**, 2325 (1983).

Methods in Enzymology

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Vasopressin and Oxytocin mRNA Regulation in the Rat Assessed by Hybridization with Synthetic Oligonucleotides*

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Vasopressin and oxytocin are nonapeptide hormones that regulate water metabolism and lactation, respectively. To study the regulation of the vasopressin and oxytocin genes at the mRNA level, we constructed a series of synthetic oligonucleotides, from 8 to 15 bases in length, for use in filter-blot hybridization assays (Northern blots) of hypothalamic mRNA levels and for primed synthesis of cDNAs from which we determined the nucleotide sequences of the 5′ regions of the vasopressin and oxytocin mRNAs. A 20-fold increase occurred in the amounts of the two mRNAs present in the hypothalami of rats drinking 2% saline for three weeks. In addition, the sequence analyses of the cDNAs provided the complete amino acid sequences of the NH_2-terminal signal peptides of the rat vasopressin and oxytocin precursors. Thus, synthetic oligonucleotides consisting of as few as eight nucleotides can be used to prime reverse transcription of specific cDNAs from hypothalamic RNA, and pentadecanucleotide hybridization probes readily detect changes in levels of vasopressin and oxytocin mRNAs in response to osmotic stress.

Vasopressin and oxytocin are nonapeptide hormones[1] synthesized in the magnacellular neurons of the hypothalamus in the form of two large polyprotein precursors, PreProVP-Np[2] and PreProOT-Np, each of which consists of the nonapeptide covalently linked at its COOH terminus to a sequence-specific neurophysin (1–6). In the vasopressin precursor, the COOH terminus of neurophysin is additionally linked to a glycopeptide (2, 3, 5, 6). The two hormones, noncovalently attached to their specific neurophysins, are transported within axons to the posterior pituitary, where they are released in response to changes in the osmolality of the extracellular fluid, as well as by complex neuronal influences (2, 3). The principle actions of oxytocin and vasopressin are to stimulate lactation and renal water conservation, respectively.

We wish to study the regulation of the expression of the vasopressin and oxytocin genes in the rat. To accomplish these studies, it is useful to have available specific cDNAs with which to evaluate the levels of cellular RNAs using hybridization techniques. As such, we have chemically synthesized oligonucleotides that are complementary to the oxytocin and vasopressin mRNAs. We report that utilization of ^{32}P-labeled synthetic pentadecanucleotides in hybridization blots of hypothalamic RNA prepared from rats fed 2% saline in their drinking water for three weeks results in a 20-fold increase in both vasopressin and oxytocin mRNA levels. In addition, nucleotide sequence analysis of the labeled cDNAs reverse transcribed from hypothalamic mRNA using the pentadecanucleotides as hybridization primers provided the amino acid sequences of the NH_2-terminal signal peptides of both PreProVP-Np and PreProOT-Np in the rat.

EXPERIMENTAL PROCEDURES

Preparation of Polyadenylated mRNA—Adult female rats (Long-Evans), 200–225 g, body weight, were given either water or 2% saline to drink for three weeks. The rats were sacrificed by decapitation and hypothalami were immediately removed and stored in liquid nitrogen. Hypothalamic mRNA was prepared by the method of Chirgwin *et al.* (8). Polyadenylated mRNA was isolated from total cellular RNA by affinity chromatography on oligo(dT)-cellulose (T-3, Collaborative Research) (9).

Preparation of Synthetic Oligodeoxyribonucleotides—Four separate octanucleotides and a mixed pentadecanucleotide were synthesized by the triester method (10). The desired oligonucleotides were separated from smaller products by reverse phase high pressure liquid chromatography on C18 resin (Waters Associates) (11).

5′-End Labeling of Synthetic Oligonucleotides Using [γ-^{32}P]ATP—Twenty pmol of oligonucleotide were labeled at the 5′ end using a 10% molar excess of [γ-^{32}P]ATP (Amersham, 5000–7000 Ci/mmol) (12), to a final specific activity of 4–6 × 10^6 cpm/pmol, and stored frozen at −20 °C at a final concentration of 0.5 pmol/μl.

Reverse Transcription of cDNA from Hypothalamic mRNA Using Labeled Oligonucleotide—For analytical purposes, 1 μg of mRNA, 2 pmol of ^{32}P-end-labeled oligonucleotide, 100 mM KCl, 0.1 mM EDTA, 100 mM Tris-HCl (pH 8.3) in a final volume of 10 μl were heated to 90 °C for 2 min and placed at 4 °C for 2 h. The reaction was adjusted to 10 mM $MgCl_2$, 10 mM dithiothreitol, 500 μM each of the four deoxyribonucleotides, 100 μg/ml of bovine serum albumin, 750 units/ml of reverse transcriptase (Life Sciences) in a final volume of 20 μl. The reaction was carried out successively at 4 °C for 5 min, 23 °C for 10 min, and 41 °C for 45 min, followed by extraction with phenol/chloroform, precipitation in ethanol, digestion in alkali, and denaturation as described by Noyes *et al.* (13). For preparative purposes, reactions were scaled up by a factor of 10.

Polyacrylamide Gel Electrophoresis and Nucleotide Sequence Analysis of cDNAs—The cDNAs of reverse transcription reactions were separated by electrophoresis on slab gels (0.04 × 40 × 15 cm) containing 10 mM Tris, 10 mM borate, 0.2 mM EDTA (pH 7.5), 7 M urea, and 5% polyacrylamide (14). Autoradiography was carried out at −70 °C using an enhancing screen (Lightning Plus, Kodak). Relevant cDNAs were eluted from the gel (15) and sequenced (16).

RNA Hybridization (Northern Blot Analysis)—Northern blot analyses were performed using the procedure of Thomas (17). Briefly, RNA was denatured in 1 M glyoxal and 50% Me_2SO, electrophoresed through a 1.5% agarose gel, transferred to nitrocellulose in 20 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.4) and baked (17). The filters were prehybridized for 1 h at 35 °C in 4 × SSC, 0.1% sodium dodecyl sulfate, 10 μg/ml of heat-denatured salmon sperm DNA, 1 × Denhardt's solution (0.2% polyvinylpyrrolidone (M_r = 40,000), 0.2% Ficoll (M_r = 400,000), 0.2% bovine serum albumin), 10 mM EDTA, after which the 5′-end-labeled pentadecamer, 1 × 10^6 cpm/ml, was added. Hybridization was for 48 h at 35 °C, followed by four 5-min washes using 5 × SSC, 0.1% sodium dodecyl sulfate at

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[1] Vasopressin, Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly; Oxytocin, Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly.

[2] The abbreviations used are: PreProVP-Np, preprovasopressin-neurophysin; PreProOT-Np, preprooxytocin-neurophysin; VP, vasopressin; OT, oxytocin; Np, neurophysin.

4 °C. The filters were air dried and autoradiography was performed for 24 to 48 h at −70 °C using an enhancing screen. In the same gels, unlabeled ribosomal RNAs were used as size markers. The amount of radioactivity in an RNA band was semiquantitatively assessed by densitometric scanning of the autoradiogram (Quick-Scan, Helena Laboratories). With this method, the density of the hybrid image is linear with the amount of RNA analyzed within the range of exposure studies (18).

RESULTS

Four octadeoxyribonucleotides, 5'GG$\overset{A}{G}$CA$\overset{A}{G}$TT3', complementary to oxytocin and vasopressin (Asn-Cys-Pro) were synthesized. This tripeptide is invariant among the entire vasopressin-related family of peptides (1), and the codon degeneracy for this tripeptide is lower than that of any other region of the nonapeptides. These four octanucleotides, labeled with ^{32}P, were used to reverse transcribe cDNA after hybridization to polyadenylated RNA isolated from hypothalami of water-fed rats (control rats) and from rats given 2% saline to drink for three weeks (salt-fed rats). With three of the four labeled octanucleotides, no differences were found in the electrophoretic patterns of the cDNAs reverse transcribed from mRNA prepared from the hypothalami of control compared to salt-fed rats (data not shown). However, one octanucleotide (5'GGGCAGTT3') reverse transcribed several cDNAs from the mRNA of salt-fed rats that were not found in the comparable reverse transcription reaction of mRNA from control rats (Fig. 1).

Partial nucleotide sequences were determined from several cDNAs specific to the mRNA isolated from salt-fed rats.

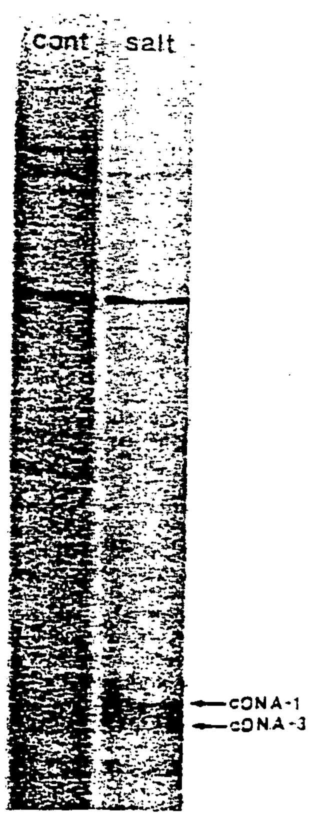


FIG. 1. **Autoradiogram of gel electrophoresis is of cDNAs primed with the octanucleotide, 5'GGGCAGTT3', using hypothalamic mRNA isolated from rats given water (*cont*) or 2% saline (*salt*) to drink for three weeks.** *Arrows* indicate two of the most prominent cDNAs (cDNA-1 and cDNA-3) specifically primed with mRNA isolated from salt-fed rats.

Although the amounts of radioactivity in the cDNAs were low (about 2000 cpm), they were sufficient to establish unambiguously that one of the cDNAs (cDNA-1) (5'GGGCAGTTCTGGAAGTAGCA3') was complementary to VP-Np mRNA (5'... $\overset{Cys}{UGC}$ $\overset{Tyr}{UAC}$ $\overset{Phe}{UUC}$ $\overset{Gln}{CAG}$ $\overset{Asn}{AAC}$ $\overset{Cys}{UGC}$ $\overset{Pro}{CC}$...), and a faster migrating cDNA (cDNA-3) (5'GGGCAGTTCTGGATGTAGCA3') was complementary to OT-Np mRNA 5'... $\overset{Cys}{UGC}$ $\overset{Tyr}{UAC}$ $\overset{Ile}{AUC}$ $\overset{Gln}{CAG}$ $\overset{Asn}{AAC}$ $\overset{Cys}{UGC}$ $\overset{Pro}{CC}$...3' (Fig. 1). The two sequences differ only in the substitution of an A for a U in the first position of the codons for phenylalanine/isoleucine. None of the remaining cDNAs specific to the mRNA isolated from salt-fed rats yielded nucleotide sequences corresponding to vasopressin or oxytocin.

This sequence information was used in the synthesis of a mixed pentadecanucleotide (5'GTTCTGG$\overset{A}{A}$TGTAGCA3') complementary to both VP-Np and OT-Np mRNA. The two pentadecamers in the mixture were separated one from the other by high pressure liquid chromatography: vasopressin-15, 5'GTTCTGGAAGTAGCA3', complementary to VP-Np mRNA and oxytocin-15, 5'GTTCTGGATGTAGCA3', complementary to OT-Np mRNA.

Hypothalamic mRNAs prepared from control and salt-fed rats were analyzed by agarose gel electrophoresis, filter blotting, and hybridization with the mixed pentadecamers (Northern blot). These analyses revealed two hybridizable RNAs, of approximately 750 and 680 bases, detected in the mRNA from the salt-fed rats (Fig. 2a, *lane 2*). Although individual quantitation of each mRNA was precluded by their similar sizes, taken together, the hybrid image of these mRNAs was 20 times less intense in the mRNA from control rats (Fig. 2, *a* and *b*). This same blot was washed free of radioactivity and rehybridized consecutively to oxytocin-15 and vasopressin-15. Rehybridization showed that the larger mRNA of 750 bases represented VP-Np mRNA, whereas the mRNA of 680 bases corresponded to OT-Np mRNA (Fig. 2a, *lanes 3* and *4*).

The mixed pentadecamer was next used to prime the reverse transcription of polyadenylated hypothalamic RNA isolated from salt-fed rats (Fig. 2c). Three cDNAs of approximately 150 bases were reverse transcribed, each differed from the next by two bases (cDNA-1, cDNA-2, cDNA-3). These three cDNAs were isolated and sequenced. cDNA-1 and cDNA-2 were identical in sequence and corresponded to the 5'-end of VP-Np mRNA, while cDNA-3 was complementary to the 5'-end of OT-Np mRNA (Fig. 3). OT-Np mRNA contains a 5' untranslated region of approximately 72 bases, followed by a nineteen amino acid signal, or leader, sequence commencing with a single methionine and containing a central region of hydrophobic amino acids characteristic of signal sequences found at the NH_2 terminus of proteins destined for secretion (19). The signal sequence likely terminates in alanine, and is immediately followed by cysteine, the first amino acid of oxytocin.

VP-Np mRNA consists of a 5' untranslated region of approximately 73 bases, which is followed by three possible initiator methionine codons (at codon positions −22, −20, and −19), all in phase with the rest of the precursor (Fig. 3). Compared with OT-Np mRNA, the two additional sites of possible initiation of translation (at codon positions −22 and −20) are due to base substitutions resulting in the additional AUG codons in VP-Np mRNA. Otherwise, the 5'-ends of rat OT-Np and VP-Np mRNAs differ at 27 of 90 bases, resulting in differences of 9 amino acids. The nucleotide sequences of cDNA-1 and cDNA-2 are identical in the region sequenced

FIG. 2. **Northern blot analysis and reverse transcription of hypothalamic mRNAs using pentadecanucleotides.** *a*, RNA hybridization assay (Northern blot) of hypothalamic mRNA isolated from control rats (*lane 1*) or salt-fed rats (*lanes 2–4*) hybridized with a mixed pentadecamer, *VP/OT-15*, complementary to both VP-Np and OT-Np mRNAs (*lanes 1* and *2*), *VP-15*, complementary to VP-Np mRNA (*lane 3*), and *OT-15*, complementary to OT-Np mRNA (*lane 4*). Unlabeled ribosomal RNAs were used as molecular weight markers. (Exposure time ~48 h.) The hybrid images corresponding to the mRNA of control rats (*lane 1*) do not reproduce well in the photograph. *b*, densitometric scan of autoradiogram of Northern blot displayed in *a*, *lane 1* (control), exposed for 96 h, and *lane 2* (salt), exposed for 48 h. *Number above each tracing* corresponds to integrated area corrected for exposure time, in arbitrary units, under each curve. *c*, autoradiogram of gel electrophoresis of cDNAs primed with the mixed pentadecamer, 5'GTTCTGGATGTAGCA3' (A above the T), using hypothalamic mRNA isolated from salt-fed rats.

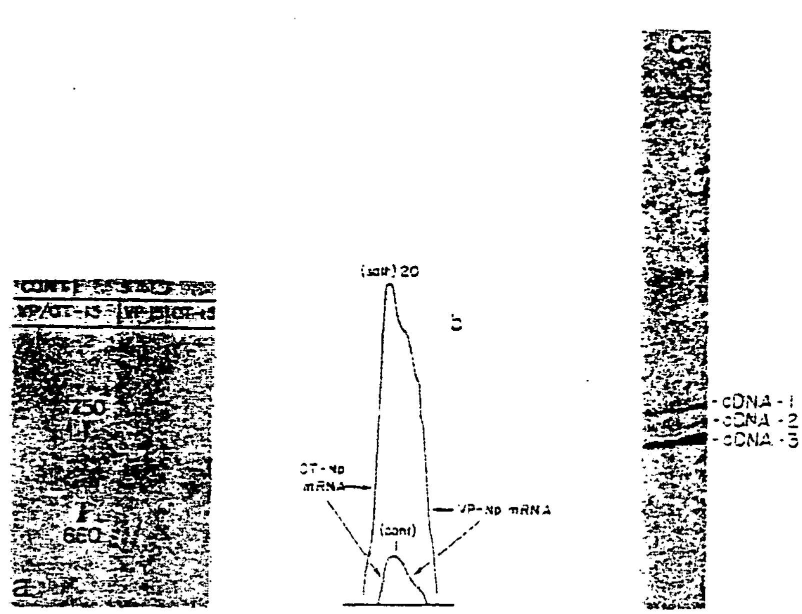
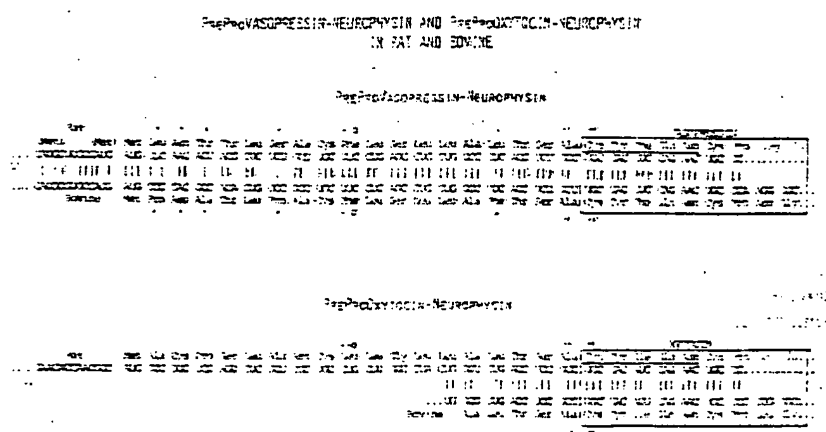


FIG. 3. **Comparison of partial nucleotide sequence of PreProVP-Np and PreProOT-Np in rat and bovine.** Deduced amino acid sequence is shown immediately adjacent to nucleotide sequence; residues in *italics* were obtained from the sequences of vasopressin and oxytocin. *Boxed areas* indicate vasopressin and oxytocin. *Arrows* underlie portions of sequence complementary to the synthetic pentadecamer used to prime cDNA reverse transcription. *Numbers* indicate amino acid positions with position +1 corresponding to the first amino acid of vasopressin or oxytocin. *Lines* indicate identical nucleotides in rat and bovine sequences. *Asterisks* indicate amino acid differences between rat and bovine proteins.



and differ only by two bases in length. It is possible that cDNA-2 arose by premature termination of the reverse transcription of VP-Np mRNA, whereas cDNA-1 represents a full-length transcript.

Comparison of the sequences of the 5'-ends of rat OT-Np mRNA and VP-Np mRNA with their bovine counterparts (3, 4) reveals that the two genes are highly conserved (Fig. 3). Rat and bovine OT-Np mRNA differ in only 3 bases in their 5' nucleotide sequences and have no differences in their deduced amino acid sequences. Bovine VP-Np mRNA contains a single initiator methionine codon beginning at amino acid −19, whereas in the rat there are three such codons within twelve bases of each other. In the VP-Np mRNA regions sequenced, there are 17 base differences between the two species, resulting in five conservative amino acid differences. The two VP-Np mRNAs differ most in the region encoding the NH_2 termini region of their signal sequences, are very similar in the COOH-terminal portion of their signal sequences, and are identical in the region encoding vasopressin itself.

DISCUSSION

The results of these studies demonstrate that synthetic oligonucleotides as small as 3 bases can be used successfully to identify and partially characterize the primary structures of hypothalamic mRNAs encoding specific proteins. In principle, this approach could be used to characterize mRNAs encoding any protein in which only three contiguous amino acids were known. However, because of the high degree of nonspecific hybridization which occurs with such short oligonucleotides, the success of this approach depends upon the ability to compare the oligonucleotide-primed synthesis of cDNAs using mRNA from control animals with that from animals in which mRNA levels can be stimulated. Since chronic dehydration, or salt loading, is known to be a potent stimulus of vasopressin and oxytocin secretion in the rat (7), we reasoned that it might result in increased mRNA levels encoding these proteins. This in fact was the case, and allowed us to correctly identify cDNAs encoding PreProOT-Np and PreProVP-Np.

The relatively low efficiency of hybridization of the octanucleotides to their complementary mRNAs allowed elucidation of only a short sequence of these mRNAs, but this was sufficient to provide information for the synthesis of larger pentadecanucleotides. These larger nucleotides primed the synthesis of cDNAs at a much higher efficiency. This "primer walking" approach allowed us to obtain the sequences of most of the 5′ ends of the mRNAs encoding rat PreProVP-Np and PreProOT-Np, starting with oligonucleotides encoding less than three amino acids.

Comparison of the 5′ ends of the mRNAs encoding rat PreProVP-Np and PreProOT-Np with the bovine mRNAs reveals that the two genes are highly conserved and arose by a duplication of a common ancestral gene (20). Bovine PreProVP-Np has only one possible initiator methionine (at amino acid −19) whereas in the rat there exist three possible sites for initiation of translation (at codon positions −22, −20, and −19). It will be of interest to determine at which of these sites initiation of translation actually occurs in light of the hypothesis that the AUG codon closest to the 5′-end of an mRNA usually functions as the initiator codon (21).

The pentadecanucleotides were of sufficient length to be used as hybridization probes for the analyses of the specific mRNAs on Northern blots (22). In our preliminary hybridization analyses, we found a 20-fold increase in the amounts of hybridizable oxytocin and vasopressin mRNAs isolated from hypothalami of rats given 2% saline to drink for three weeks. This is consistent with findings that prolonged dehydration, or salt feeding, is associated with marked stimulation of blood levels of vasopressin and oxytocin (23), as well as with the depletion of posterior pituitary stores of these hormones (24). Whether the increased mRNA levels are due to increased transcription or decreased degradation of the mRNAs, or both circumstances, remains to be determined.

Each of the two pentadecamers, VP-15 and OT-15, differing in sequence by only one base, hybridized uniquely to its complementary mRNA, allowing the identification of the 750-base mRNA encoding PreProVP-Np and the smaller 680-base mRNA encoding PreProOT-Np. The sizes of these mRNAs in the rat agree well with those reported for the corresponding bovine mRNAs (3, 4). The ability to discriminate between similar mRNAs using short oligonucleotides differing by only one base has also been used successfully in analyses of closely related mutant globin genes (25, 26).

These pentadecanucleotides will be useful in more extensive and detailed analyses of the regulation of vasopressin and oxytocin gene expression. They will also be useful as hybridization probes to select full-length cloned recombinant cDNAs prepared from the rat hypothalamus, which will provide more complete nucleotide sequence of the vasopressin and oxytocin precursors in the rat.

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Addendum—After this work was submitted for publication, a partial sequence of the gene encoding rat VP-Np was published. The two sequences are in agreement (27).

REFERENCES

1. Bisset, G. W. (1976) in *Peptide Hormones* (Parsons, J. A., ed) pp. 145–177, Macmillan Press, Ltd., London
2. Brownstein, M. J., Russel, J. T., and Gainer, H. (1980) *Science (Wash. D. C.)* **203**, 449–458
3. Land, H., Schutz, G., Schmale, H., and Richter, D. (1982) *Nature (Lond.)* **295**, 299–303
4. Land, H., Grez, M., Ruppert, S., Schmale, H., Rehbein, M., Richter, D., and Schutz, G. (1983) *Nature (Lond.)* **302**, 342–344
5. Russel, J. T., Brownstein, M., and Gainer, H. (1981) *Neuropeptides* **2**, 59–65
6. Ivell, R., Schmale, H., and Richter, D. (1981) *Biochem. Biophys. Res. Commun.* **102**, 1230–1236
7. Jones, C. W., and Pickering, B. T. (1969) *J. Physiol.* **203**, 449–458
8. Chirgwin, J. M., Przybyla, A., MacDonald, R., and Rutter, W. (1979) *Biochemistry* **24**, 5294–5299
9. Aviv, H., and Leder, P. (1972) *Proc. Natl. Acad. Sci. U. S. A.* **69**, 1408–1412
10. van Boom, J. H., van Deursen, P. H., and Burgers, P. M. J. (1976) *Tetrahedron Lett.* 369–372
11. Wallace, R. B., Johnson, M., Hirose, T., Miyake, T., Kawashima, E., and Itakura, K. (1981) *Nucleic Acids Res.* **9**, 879–894
12. Wallace, T. B., Shaffer, J., Murphy, R., Bonner, J., Hirose, T., and Itakura, K. (1979) *Nucleic Acids Res.* **6**, 3543–3557
13. Noyes, B. E., Mevarech, M., Stein, R., and Agarwal, K. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 1770–1774
14. Maxam, A., and Gilbert, W. (1980) *Methods Enzymol.* **65**, 499–560
15. Southern, E. (1979) *Methods Enzymol.* **68**, 152–176
16. Maxam, A., and Gilbert, W. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 560–564
17. Thomas, P. S. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77**, 5201–5305
18. Heinrich, G., Kronenberg, H. M., Potts, J. T., Jr., and Habener, J. F. (1983) *Endocrinology* **112**, 449–458
19. Majzoub, J. A., Rosenblatt, N., Fennick, B., Maunus, R., Kronenberg, H. M., Potts, J. T., Jr., and Habener, J. F. (1980) *J. Biol. Chem.* **255**, 11478–11483
20. Archer, R. (1980) *Proc. R. Soc. Lond. B Biol. Chem.* **210**, 21–43
21. Kozak, M. (1980) *Cell* **22**, 7–8
22. Comb, M., Herbert, E., and Crea, R. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79**, 360–364
23. Sinding, C., Robinson, A., and Seif, S. (1980) *Endocrinology* **107**, 755–760
24. Negro-Vilar, A., and Samson, W. (1979) *Brain Res.* **169**, 585–589
25. Orkin, S., Markham, A., and Kazazian, H., Jr. (1983) *J. Clin. Invest.* **71**, 775–779
26. Conner, B. J., Reyes, A., Morin, C., Itakura, K., Teplitz, R., and Wallace, B. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80**, 278–282
27. Schmale, H., Heinsohn, S., and Richter, D. (1983) *Eur. Mol. Biol. Org. J.* **2**, 763–767

1: Proc Natl Acad Sci U S A. 1979 Oct;76(10):5036-40.

Related Articles, Links

Construction and selection of recombinant plasmids containing full-length complementary DNAs corresponding to rat insulins I and II.

Chan SJ, Noyes BE, Agarwal KL, Steiner DF.

We have used a synthetic deoxydecanucleotide to generate an insulin-specific cDNA probe suitable for selecting transformants that contain nearly full-length cDNAs corresponding to the mRNAs coding for rat insulins I and II. Double-stranded cDNA was synthesized from x-ray-induced rat insulinoma poly(A)-RNA, inserted in pBR322 plasmid DNA by the homopolymeric tailing technique, and cloned in Escherichia coli chi 1776. Colony hybridization with oligonucleotide-primed cDNA yielded 16 positive clones of which 7 corresponded to rat insulin I mRNA and 9 to rat insulin II mRNA. Restriction endonuclease maps of representative clones of each group indicated that these contained the complete coding sequences, as was confirmed by nucleotide sequence analysis of the 5' region of the cloned DNA for rat insulin II. Nucleotide sequence analysis also established the amino acid sequence of the prepeptide of rat preproinsulin II. Comparison of the amino acid sequence of the prepeptides of rat preproinsulin I and II shows that three conservative amino acid substitutions have occurred in this region of the molecule.

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